

1 *Post-drought conditions and hydraulic dysfunction determine tree resilience and mortality*  
2 *across Mediterranean Aleppo pine (*Pinus halepensis*) populations after an extreme drought*  
3 *event*

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14 Hydraulic dysfunction affect post-drought resilience

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**Abstract**

Drought-related tree mortality is a global phenomenon that currently affects a wide range of forests. Key functional variables on plant hydraulics, carbon economy, growth and allocation have been identified and play a role in tree drought responses. However, tree mortality thresholds based on such variables are difficult to identify, especially under field conditions. We studied several Aleppo pine populations differently affected by an extreme drought event in 2014, with mortality rates ranging from no mortality to 90% in the most severely affected population. We hypothesized that mortality is linked with high levels of xylem embolism, i.e. hydraulic dysfunction, which would also lead to lower tree resistance to drought in subsequent years.

48 Despite not finding any among-populations differences in the vulnerability curves to xylem  
49 embolism, there were large differences in the hydraulic safety margin and the hydraulic  
50 dysfunction level. High mortality rates were associated with a negative hydraulic safety margin  
51 when xylem embolism reached values over 60%. We also found forest weakening and post-  
52 drought mortality related to a low hydraulic water transport capacity, reduced plant growth, low  
53 carbohydrate contents and high pest infestation rates. Our results highlight the importance of  
54 drought severity and the hydraulic dysfunction level on pine mortality, as well as post-drought  
55 conditions during recovery processes.

56 **Keywords:** forest decline, drought legacy, xylem cavitation, biotic attacks, global change, *Pinus*  
57 *halepensis*.

58

## 59 **Introduction**

60 Forest ecosystems are environmentally and economically important for the many ecosystem and  
61 environmental services that they provide (Hanewinkel et al. 2013). According to the ongoing  
62 global change, projected climate scenarios predict an increase in the mean annual temperatures  
63 and decreases up to 30% in precipitations in the Mediterranean Region (Cramer et al. 2018). These  
64 environmental conditions will increase the likelihood of extreme events, such as hotter droughts  
65 with forest dieback episodes (Niu et al. 2014). The tolerance limits for species survival and legacy  
66 effects after drought events are outstanding factors that trigger changes in forest composition,  
67 species diversity, hydrological function and potential carbon storage (Brodrribb et al. 2020,  
68 Trambly et al. 2020). Thus identifying the variables associated with mortality and their  
69 corresponding thresholds should increase our capacity to forecast drought-induced mortality  
70 (Camarero 2021, Rowland et al. 2021).

71 Plants' capacity to cope with drought is associated with both morphological and physiological  
72 traits, especially with those related to water supply to leaves through the xylem (Hernández et al.  
73 2010, Torres-Ruiz et al. 2017). Vulnerability to xylem embolism and the hydraulic safety margins

74 (HSM) of trees have emerged as key parameters to evaluate plant resistance to drought because  
75 they are directly linked with mechanisms of tree decline and mortality (Hammond et al. 2019,  
76 Brodribb et al. 2020). Both functional variables are related to xylem hydraulic failure, which  
77 affects plants' capacity to move water from soil to leaves, among other plant physiological  
78 functions (Vilagrosa et al. 2010).

79 Previous studies reported that hydraulic dysfunction (i.e. loss of hydraulic conductivity, PLC)  
80 above 60% triggers major mortality events in conifers (Brodribb et al. 2009, Adams et al. 2017).  
81 However, recent studies by Hammond et al. (2019) and Mantova et al. (2021) set this threshold  
82 for conifers at more than 70% hydraulic conductivity loss by determining greater tolerance than  
83 previously reported, which reveals that no consensus about this relevant issue has been reached.  
84 HSM is considered an estimation of plants' capacity to protect their vascular transport systems  
85 from xylem embolism (Meinzer et al. 2009). This parameter can be defined as the difference  
86 between trees' minimum water potential ( $P_{\min}$ ) and the xylem pressure at which 50% of hydraulic  
87 conductance is lost ( $P_{50}$ ; Choat et al. 2018). High HSM values, i.e wider margins, represent the  
88 safety edge of the threshold, where xylem is protected from embolism risk, while plants are  
89 considered to operate under risky conditions when HSM values come close to zero (Martin-  
90 StPaul et al. 2017).

91 Apart from the impact of drought on the level of hydraulic dysfunction(i.e. PLC) in plants, other  
92 co-occurring processes like carbohydrate depletion and pest outbreaks seem to be directly linked  
93 with post-drought tree weakening (McDowell & Sevanto 2010). Severe stomatal closure when  
94 exposed to water stress conditions (Creek et al. 2020) reduces trees' reserves of non-structural  
95 carbohydrates (NSC) due to limited  $CO_2$  fixation. This reduction in NSC reserves can be lethal  
96 because it diminishes trees' ability to tolerate drought and to resume post-drought growth  
97 (Martínez-Vilalta et al. 2016, Lloret et al. 2018). In addition, when drought conditions are  
98 accompanied by high temperatures, the possibility of pest outbreaks increases because of greater  
99 insect activity and high reproduction rates (Choat et al. 2018). Biotic agents like plant pathogens  
100 and insects may intensify negative drought effects and reduce plant vitality to surpass drought  
101 conditions and to recover healthy conditions (Gaylord et al. 2013, Gaylord et al. 2015, Hartmann

102 et al. 2015). The ultimate consequence is tree weakening, which may lead to higher tree mortality  
103 after drought or in subsequent years (Cailleret et al. 2017, Morcillo et al. 2019).

104

105 In the Mediterranean Basin, Aleppo pine (*Pinus halepensis* Mill.) is the dominant conifer tree  
106 species that covers around 2.5 million hectares worldwide (Ne'eman and Trabaud 2000). It  
107 frequently lives under extremely harsh conditions (Pausas et al. 2004, Del Campo et al. 2007) and  
108 is considered a well-adapted species that can withstand drought via several morpho-functional  
109 mechanisms, including growth adaptations (Klein et al. 2013, Taïbi et al. 2017, Vicente et al.  
110 2018). However, the increasingly dry conditions that have appeared in recent years have led  
111 Aleppo pine forest populations to increasingly decline (García de la Serrana et al. 2015,  
112 Greenwood et al. 2017), which is sometimes exacerbated by interactions with other biotic factors  
113 (Benavides et al. 2013, Morcillo et al. 2019). Normally the persistence of simultaneous interacting  
114 post-drought decline processes makes it difficult to disentangle the role of the abiotic and biotic  
115 factors in these decline processes and to, therefore, explain decline and mortality.

116 The aim of the present study was to identify the key functional variables capable of explaining  
117 the decline and mortality observed in several Aleppo pine populations during the 2014 drought,  
118 and the post-drought recovery or tree die-off 2 years after the extreme drought event. To do so,  
119 we measured several morphological, physiological and hydraulic variables to assess: i) water  
120 stress dynamics at predawn and midday, vulnerability to xylem embolism (i.e. vulnerability  
121 curves), the hydraulic dysfunction level (i.e. PLC) as a consequence of drought intensity, and the  
122 magnitude of HSM for each population during and after drought; ii) post-drought carbohydrate  
123 dynamics and pine growth; iii) the incidence of pine pests (i.e. bark beetles and pine processionary  
124 moth). This allowed us to evaluate and establish relations between tree functional variables and  
125 their thresholds with the observed mortality levels. We hypothesized that drier populations would  
126 be exposed to lower water potentials and, as vulnerability to embolism among populations is  
127 typically unrelated to aridity (e.g. Rosas et al., 2019 for this species), we expected mortality to be  
128 related to higher PLC and narrower HSMs in drier populations. Consequently, these drought

129 legacy effects could also entail growth decline, carbohydrate depletion or increased pest diseases  
130 and, finally, new mortality events in the most affected populations.

131 Our findings will provide a better understanding of how trees will respond and overcome the  
132 increased frequency of hotter droughts in forthcoming decades. Studies conducted under natural  
133 conditions will be valuable because they represent real conditions, which are scarcely reported  
134 in the common literature, especially in response to varying environmental conditions like those  
135 in the present study (Hartmann et al. 2018, Rosas et al. 2019).

136

## 137 **Materials and Methods**

### 138 **Study sites description and experiment design**

139 The study was conducted from late autumn 2015 (November) to late winter 2017 (January) in  
140 four *Pinus halepensis* populations reforested during the 1945-1965 period. They are located in  
141 SE Spain on a north-south gradient, which also corresponds to an aridity gradient. From south to  
142 north, populations are Orihuela, Albaterra, San Juan and Catí (*see Table S1 available as*  
143 *Supplementary Data at Tree Physiology Online*). All the populations were S-SE-exposed. Soil  
144 developed on marly bedrock, characterized by being shallow with high carbonate content, basic  
145 pH, and low organic matter content and fertility levels. In each population, measurements were  
146 taken in seven selected individuals that showed no signs of decline after the extremely intense  
147 drought in 2014, whose size was average for the stand (*Table S1 available as Supplementary*  
148 *Data at Tree Physiology Online*). These pine populations have already been studied in García de  
149 la Serrana et al. (2015). Some field results, such as minimum water potentials and stand mortality  
150 from that study, were also employed in the present study.

### 151 **Climate data and drought characterization**

152 The annual mean precipitation and historical averaged temperature for each population are shown  
153 in Table S1 (*available as Supplementary Data at Tree Physiology Online*). The average values  
154 were assigned from the nearest thermo-pluviometric stations (the AEMET weather stations

155 network) and values were the averages for the 1983-2012 period. According to these values, all  
156 the forests are located within the semiarid range (200-350 mm/year), except for Catí, which is  
157 located in a dry climate (350-600 mm, Pérez-Cueva 1994). The mean temperature recorded for  
158 the study period (2014-2016) was 19.1°C for the Orihuela and San Juan populations, 19.2°C for  
159 Albatera and 15.1°C for the Catí population.

160 The Standardized Precipitation Evapotranspiration Index (SPEI) was estimated for drought  
161 characterization purposes on two time scales: 6 and 12 months. The SPEI is a multiscalar drought  
162 index based on climatic water balance anomalies in relation to the normal conditions at a given  
163 site, which determines the onset and magnitude of droughts (Vicente-Serrano et al. 2010). The  
164 SPEI was calculated as the difference between the precipitation and potential evapotranspiration  
165 that accumulated from 2000 to 2016 using the climate data from the nearest meteorological  
166 stations to each site according to a site's coordinates. Values were standardized after fitting log-  
167 logistic distribution to data. Thus the SPEI expressed standard deviations following a standard  
168 Gaussian distribution (Beguería et al. 2014).

### 169 **Pine mortality and pest infestation estimations**

170 Pine mortality was assessed by counting the number of living pines and those that had recently  
171 died (i.e., dead individuals after 2014) in a large stand (i.e. minimum of 0.5 ha) per population.  
172 Censuses were carried out at the end of each year. Trees were considered recently dead if all their  
173 leaves were brown. After 1 year, field observations showed that brown leaves had turned gray  
174 and these trees were not considered to be recently dead.

175 The pest infestation monitoring data (bark beetle, BB, of the genera *Tomicus* spp. and  
176 *Orthotomicus* spp, and pine processionary moth at the end of larval development; PPM,  
177 *Thaumetopoea pityocampa*) were provided by the Forestry Services (Generalitat Valenciana,  
178 Regional Valencian Government). Qualitative categories (0, absence; 1, some infestation in  
179 isolated individuals located on the stand border; 2, many individuals infested on the stand border;  
180 3, many individuals infested on the stand border and some infestation at the center of the stand;  
181 4, high infestation on the stand border and partly infested at the center of the stand; 5, a generally  
182 highly infested stand) were transformed into infestation percentages (i.e., no infestation, 0%; low

183 infestation, ~20%; moderate infestation, ~40%; high infestation, ~60%; very high infestation,  
184 ~80%; total infestation, 100%) to include these data in the statistical analysis.

185 In order to determine if mortality was linked with BB attacks, we specifically evaluated the  
186 impact of BB by counting the number of dead pines affected by these insects in each population.  
187 Degree of infestation was determined by making direct observations of dead pines during  
188 mortality censuses. A tree was considered severely affected when the number of holes  
189 surrounding the trunk exceeded 100 and were distributed all over the trunk ring, which was then  
190 considered to denote irreversible phloem degradation (Forest Services, pers. comm.).  
191 Accordingly, entrance and exit holes were used to make common estimates of attack intensity  
192 (e.g., Borkowski and Skrzecz. 2016, Jaime et al. 2019). Tree bark was stripped to confirm the  
193 high BB infestation level. This methodology has been previously applied (Garcia de la Serrana  
194 et al. 2015, Morcillo et al. 2019).

#### 195 **Water potential dynamics and hydraulic measurements**

196 Water potential was used to determine the maximum degree of stress that trees suffered during  
197 the post-drought period ( $P_{min}$ ). Water potential was measured at predawn ( $\Psi_{pd}$ ) and midday  
198 ( $\Psi_{md}$ ) in January, June, July, September and November 2016, and in January 2017. Three  
199 terminal twigs per individual were selected from the middle of the tree canopy, which was  
200 southerly exposed. Twigs were placed inside a polyethylene bag along with a piece of wet paper  
201 to avoid transpiration. They were stored in a portable cooler to avoid dehydration. Measurements  
202 were taken within 15 minutes after sampling by using a Schölander pressure chamber (Model  
203 1000, Pressure Chamber, Instrument, PMS Instrument Company, Albany, Oregon, USA).

204 Xylem vulnerability to embolism was determined at the shoot level in the same trees used for the  
205 previous measurements. Thus from the same mature trees at each site, one 0.5-0.7 m long x 0.5-  
206 1 cm in diameter branch per tree was sampled early in the morning (8-9 am solar time) by using  
207 pole pruners. Branches were immediately wrapped in moist paper and placed inside black airtight  
208 plastic bags to reduce water loss. Samples were sent to the PHENOBOIS platform (Bordeaux,  
209 France) and refrigerated until measurements were taken. At the lab, branches were recut under



210 water with a razor blade to a standard length of 27 cm and bark was removed to prevent resin  
 211 contamination during measurements. Finally, xylem vulnerability to embolism was determined  
 212 by the Cavitron technique (Cochard et al. 2005), based on centrifuging samples while measuring  
 213 their hydraulic conductance. This method allows xylem hydraulic conductivity to be measured  
 214 under negative pressure. Initially, the maximum hydraulic conductivity ( $K_{\max}$  in  $\text{m}^2 \text{MPa}^{-1} \text{s}^{-1}$ )  
 215 was measured at high xylem pressure (low speed) ( $P$  in MPa). Then the centrifuge rotation speed  
 216 was gradually increased by 0.5 or 1 MPa to progressively lower xylem pressure. Vulnerability to  
 217 embolism curves were generated by plotting the percentage loss of hydraulic conductivity (PLC)  
 218 at each induced negative xylem pressure. PLC was calculated according to the following  
 219 equation:

$$220 \text{ PLC} = 100(1 - K_i/K_{\max})$$

221 where  $K_{\max}$  ( $\text{m}^2 \text{MPa}^{-1} \text{s}^{-1}$ ) was the maximum hydraulic conductivity measured at low speed to  
 222 avoid inducing any cavitation event and  $K_i$  was the hydraulic conductivity measured at each  
 223 induced xylem pressure. Vulnerability curves were fitted using the equation of Pammenter and  
 224 Vander Willigen (1998):

$$225 \text{ PLC} = 100/[1 + \exp(s \times (P - P_{50}))]$$

226 where  $P_{50}$  (MPa) is the xylem pressure at which 50% hydraulic conductance was lost, and  $s$  is  
 227 the slope of the curve at the inflection point. The maximum xylem-specific hydraulic  
 228 conductivity ( $K_s$ ,  $\text{m}^2 \text{MPa}^{-1} \text{s}^{-1}$ ) was calculated as the maximum hydraulic conductivity measured  
 229 at a low speed divided by the sample's sapwood area. From the equation, we derived the xylem  
 230 water potential estimations that would cause 12% and 88% loss of conductivity ( $P_{12}$  and  $P_{88}$ )  
 231 values, where  $P_{12} = P_{50} + 50/s$  was considered the 'air entry point' (Sparks and Black 1999) and  
 232  $P_{88} = P_{50} - 50/s$  was taken as the limiting tension before the xylem became completely  
 233 nonconductive (Domec and Gartner 2001).

234 The hydraulic safety margin (HSM) for each population was calculated as the difference between  
 235 the minimum water potential ( $\Psi_{\min}$ ) reached under field conditions within a year and  $P_{50}$  was

236 determined from the vulnerability curves established in each population according to the  
237 methodology described in Delzon and Cochard (2014). The hydraulic dysfunction levels (i.e.  
238 PLC), and HSM were related to the pine mortality of each population for the 2014 and 2016  
239 records when  $\Psi_{\min}$  were recorded under field conditions at midday. The  $\Psi_{\min}$  data for the PLC  
240 and the HSM calculations in 2014 were taken from García de la Serrana et al. (2015).

#### 241 **Nonstructural carbohydrates (NSC)**

242 Nonstructural carbohydrates (NSC) were determined as soluble sugars (SS, including sucrose,  
243 glucose and fructose) and starch content in the branches collected in June 2016 and January 2017.  
244 From the same trees used for the previous measurements, a 10 cm-long branch sample with no  
245 bark was collected from the basal part of a well-developed branch in the medium-upper crown  
246 part. This part of the tree has been used in former studies and provides reliable information about  
247 NSC dynamics (Anderegg and Anderegg 2013, Rosas et al. 2013). Immediately after cutting,  
248 samples were frozen at  $-196^{\circ}\text{C}$  with liquid nitrogen in a Dewar portable container to stop  
249 enzymatic activity. At the lab, samples were transferred to a freezer and stored at  $-25^{\circ}\text{C}$ . Finally,  
250 the frozen samples were sent to a specialized laboratory (Agrolab Analítica SL). There the NSC  
251 samples were oven-dried at  $75^{\circ}\text{C}$ , ground in an electrical mill and passed through a 1-mm screen.  
252 Starch was determined by the enzymatic method (amyloglucosidase and glucose-oxidase–  
253 peroxidase) and later measured colourimetrically by a visible spectrophotometer at the 500 nm  
254 UV wavelength. Total and reductors SS were extracted by reduced ferrocyanide, and their  
255 concentration was colourimetrically determined by a visible spectrophotometer at the 540 nm  
256 UV wavelength.

#### 257 **Morphological traits**

258 Several tree morphology- and growth rate-related variables were determined to evaluate the  
259 effect of drought and post-drought on recovery capacity: the leaf mass fraction (LMF) as the ratio  
260 between the leaf dry mass and the total twig dry mass, and the leaf-to-wood area ratio ( $A_l/A_s$   
261  $_{(\text{twig})}$ ) as the ratio between leaf area and the twig section and needle length, which were measured

262 in November 2016. Three terminal twigs of ca. 3 mm in diameter were sampled per selected tree.  
263 At the lab, stem diameter (without bark) and needle length were measured in n=15 needles per  
264 twig. To determine leaf area, all the needles per twig were scanned and the projected area was  
265 estimated by using the *Medición de Objetos v. 4.2* © 1999-2000 R. Ordiales software. All the  
266 biomass per fraction was then oven-dried for 48 h at 65°C and then weighed on a precision scale.  
267 Finally, both the LMF and the  $A_l/A_s$  (twig) ratio were calculated. All these variables were used to  
268 analyze the growth parameters related to tree vigor and the ability to maintain water flow to  
269 leaves (Perez-Harguindeguy et al. 2016, Mencuccini et al. 2019).

270

### 271 **Data analysis**

272 The differences in the water potentials ( $\Psi$ ) between populations for the 2015-2017 range were  
273 analyzed by a Repeated Measures ANOVA (GLM) with Population (P) at four levels (Orihuela,  
274 Albaterra, S. Juan and Catí) as the between-subject factor, and Time (T) as the within-subject  
275 factor. The differences in the minimum water potentials (Pmin), NSC content between  
276 populations (i.e. starch and SS), LMF,  $A_l/A_s$  (twig), needle length, xylem vulnerability to embolism  
277 (P12, P50 and P88) and Ks were analysed by a General Linear Model (GLM) one-way ANOVA  
278 with one fixed factor: Population (P, four levels). Tukey's HSD (Honestly Significant  
279 Difference) tests were run for the mean pairwise multiple comparisons.

280 Finally, a redundancy analysis (RDA) was used to investigate the relation between these  
281 predictor variables (i.e. physiological, morphological variables, carbohydrate content and pest  
282 infestation) and the response variable (i.e. observed pine mortality) across populations. This  
283 analysis reduces the dimensionality of the different measured variables (Legendre and Legendre,  
284 1998). The RDA analysis allowed variation to be extracted and summarised in a series of  
285 response variables that can be explained by a set of explanatory variables. To increase our  
286 analysis power, we also included some parameters measured in 2014 (taken from García de la  
287 Serrana et al. 2015), such as pine mortality (Mort\_14), the hydraulic dysfunction level (PLC\_14)  
288 and the minimum water potential (Pmin\_14). The HSM was not included in the RDA to avoid  
289 redundancy with the PLC and Pmin. The RDA was carried out on scaled and centred data using

290 the prcomp function from the R 'stats' package. Data were transformed whenever necessary to  
291 meet the normal distribution of residuals and homoscedasticity assumptions. Data analysis were  
292 carried out by the SPSS 23.0 Statistical package (SPSS Inc., Chicago, IL, USA) and the R studio  
293 software (RStudio Team, 2015).

## 294 **Results**

### 295 **Drought intensity and mortality levels**

296 The annual precipitation in 2014 was much lower than the historical averaged values for all the  
297 sites with reductions from 30% to 50% (Fig. 1, A). During the 2014 drought event, the Orihuela,  
298 Albatera and San Juan records went below 200 mm, while Catí remained above 250 mm for the  
299 whole study period. The recorded values came closer to the historical average in the following  
300 years, but it was not until 2016 when the precipitation values equalled the historical average of  
301 the Orihuela and Catí sites. However, Albatera and San Juan remained under drier conditions  
302 compared to the historical records for the following years.

303 In addition, temperature anomalies, i.e. temperature deviations during the study period from  
304 historical records, were between 0.6°C and 1.4°C higher than the historical records, which reflects  
305 intense hot conditions (Fig.1, B).

306

307 **Place Fig. 1 here**

308

309 Based on the SPEI results, the 2014 drought was considered the severest event at the four  
310 study sites (*see Figure S1 available as Supplementary Data at Tree Physiology Online*) with  
311 values under -2. The results of the 6-month period SPEI showed a predominant presence of  
312 drought periods mixed with some milder periods. The 12-month SPEI clearly revealed an  
313 extended drought period for the next 2 years (until 2016), except in Catí, where drought  
314 intensity significantly diminished in 2015.

315 Pine mortality rates were maximum in Orihuela, with the highest mortality for 2014 (95%), which  
316 increased gradually for the next 2 years, and almost all the pines in that area died (99%) (Fig. 2,  
317 A). No dead pines were found at Catí throughout the study period. Albatera and San Juan obtained  
318 intermediate mortality values. At Albatera, 20% of the individuals died in 2014, and mortality  
319 slightly increased in the following years. Mortality at San Juan was low (c.a. 5%) in 2014, but  
320 significantly increased for the next 2 years and reached a maximum of 80% in 2016.

321

### 322 **Pest infestation**

323 Bark beetles (BB) were detected in the dead trees of the three populations for which some  
324 mortality cases were found (i.e. Orihuela, Albatera, San Juan). The impact of BB differed among  
325 populations ( $F = 11.938$ ;  $p < 0.001$ ). At Orihuela, almost all the dead trees showed BB  
326 infestations ( $92 \pm 3\%$ ). At Albatera, BB attacks were detected in 51% of all the dead trees and in  
327 only 22% at San Juan (Figure 2, B).

328 The records of pine processionary moth (PPM) presence in living pines for 2016 showed a higher  
329 degree of infestation for the San Juan population than in Albatera and Orihuela (Fig. 2, C), with  
330 no signs at Catí.

331 The analysis of both diseases showed that neither PPM nor BB was present at Catí throughout  
332 the study period. Orihuela showed a low degree of PPM infestation, but only in 2016. It was  
333 absent for the other years, but with high BB infestation levels for the whole period (2014-16).  
334 Albatera showed a low to moderate degree of PPM infestation, while San Juan was the most  
335 infested population with a high degree throughout the study period (*Table S2 available as*  
336 *Supplementary Data at Tree Physiology Online*). BB had a moderate degree of infestation at  
337 Albatera, which was high and moderate at San Juan for 2015 and 2016, respectively.

338

339 **Place Fig. 2 here**

340

341 **Intensity of water stress**

342 The persistence of low precipitation and high temperatures in the years after the 2014 extreme  
343 drought resulted in a long period with water shortages. Thus during most of our study period, the  
344  $\Psi_{pd}$  values were lower than -3 MPa, which reflect high drought stress levels (Fig. S2 and *Table*  
345 *S3 available as Supplementary Data at Tree Physiology Online*). The minimum water potential  
346 values ( $P_{min}$ ) that reflected the maximum drought stress intensity ranged from -3.2 to -6.0 MPa  
347 for 2014 (Garcia de la Serrana et al., 2015) and from -5.0 to -6.4 MPa during the 2015-2017 period  
348 (Table 1). The Orihuela population showed the most negative water potential values, below -6  
349 MPa, at midday in June 2016. Nevertheless in late 2016-2017, San Juan was the population with  
350 lowest water potential, while the other populations recovered with less stressful water status.

351

352 **Place Table 1 here.**

353

354 **Xylem vulnerability to embolism**

355 Vulnerability to xylem embolism did not significantly differ among the studied populations, with  
356  $P_{50}$  values ranging from -5.2 to -5.7 MPa (Table 1; *see Figure S3 available as Supplementary*  
357 *Data at Tree Physiology Online*). P12 and P88 followed the same trends as P50. Only xylem-  
358 specific hydraulic conductivity ( $K_s$ ) showed differences among populations (Table 1). Catí and  
359 Orihuela had the highest  $K_s$  values, while Albaterra and San Juan obtained the lowest ones for  
360 this trait.

361

362 **Nonstructural carbohydrate dynamics**

363 Significant population differences appeared for starch content for both sampling periods, but not  
364 for SS (Table 2, *see Table S4 available as Supplementary Data at Tree Physiology Online*).

365 Starch content was always higher in the Catí population, located in the most humid location, and  
366 suffered no mortality events. Starch values ranged from the lowest average value ( $0.63 \text{ mg/g}^{-1}$   
367 dw) measured at San Juan in summer 2016 to the maximum value (about  $2.44 \text{ mg/g}^{-1}$ ) measured  
368 in the Catí population for the same period (Table 2).

369

370 **Place Table 2 here**

371

372 **Morphological traits**

373 Significant differences appeared among populations for the LMF, the degree of leaf investment  
374 in relation to twig biomass. San Juan had lower LMF values than the other populations (Fig. 3;  
375 *see Table S5 available as Supplementary Data at Tree Physiology Online*). The leaf-to-sapwood  
376 area ratio ( $A_l/A_s$ ) also revealed significant differences among populations with the lowest values  
377 for San Juan, followed by Albatera. The highest values were for Catí and Orihuela. For needle  
378 length, the San Juan population presented 2 cm on average, which was a shorter length values  
379 than the other three populations that obtained mean values of around 3.5-4 cm (Fig. 4; *see Table*  
380 *S5 available as Supplementary Data at Tree Physiology Online*).

381

382 **Place Fig. 3 here**

383

384 **Functional variable thresholds associated with mortality**

385 No correlation was found between the P50 and mortality levels for each population. However,  
386 the PLC estimated from the minimum water potentials recorded in the field and the vulnerability  
387 curves for each population had values from 0 to 40%, with extreme values over 60% for the  
388 Orihuela population in 2014 and 2016 (Fig. 4, B). Mortality exponentially increased and went

389 above 90% when the PLC values exceeded 60%. Mortality at San Juan in 2016 was exceedingly  
390 high, up to 80%, while the xylem embolism values remained low that year (Fig. 4, B).

391 The HSM varied across populations and years, with negative values when drought conditions  
392 were extreme and the minimum water potential was lower than  $P_{50}$ . This was the case of the  
393 Orihuela population (Fig. 4, A). Under these conditions, mortality at Orihuela came close to  
394 100%. Although both Albatera and San Juan showed very a narrow HSM in some specific years,  
395 Albatera presented positive values with mortality rates below 40%. Despite San Juan having a  
396 high HSM value in 2014, i.e. above +1.5, it lowered to values below 0.5 by 2016 and was  
397 associated with high mortality. The Catí population showed wide HSM values throughout the  
398 period with no associated mortality.

399

400 **Place Fig. 4 here**

401

402 The RDA for the main evaluated predictor variables showed that the first two main components  
403 explained 86% of trait variation among populations (Fig. 5). The first principal component  
404 (RDA1) explained 51% of data variability, and the variables related to water status and tolerance  
405 to drought stress, such as  $P_{min}$  and the PLC, were distributed along this axis (*see Table S6*  
406 *available as Supplementary Data at Tree Physiology Online*). Hence the variables associated  
407 with water status and drought tolerance (i.e. PLC) were closely related to both the 2014 mortality  
408 and the percentage of trees infested by BB attacks (Fig. 5, *see Table S6 and S7 available as*  
409 *Supplementary Data at Tree Physiology Online*). Conversely, we found these traits to be  
410 associated with the drought stress level and inversely related to the 2014 mortality, i.e.,  $P_{min}$  during  
411 both periods, and partially SS content. Under such conditions, the Orihuela and Catí sites occupied  
412 opposite positions, and reflected the highest and lowest drought stress levels for pines.

413 The second component (RDA2, explaining 35% of the variability between variables) was related  
414 to water flow capacity (i.e.  $K_s$ ) and morphological variables, such as the ratios between leaf and



415 twig development (i.e.  $A_i/A_s$  and LMF), and partially to needle length. Inversely, the 2016  
416 mortality was closely related to the San Juan populations, which showed poor tree vitality with  
417 stunted leaf morphological development and low water flow capacity,  $K_s$ , exacerbated by PPM  
418 infestation (*Table S6 and S7 available as Supplementary Data at Tree Physiology Online*).  
419 Albaterra, with moderate mortality rates and morphological development, did not align with any  
420 of these RDA axes.

421

422 **Place Fig. 5 here**

423

#### 424 **Discussion**

425 Determining whether Aleppo pine forests will persist under increasingly limiting conditions or  
426 if they will be replaced with better adapted vegetation types in the future poses critical uncertainty  
427 for ecosystem dynamics, society and forestry managers (Batllori et al. 2020). Our results indicate  
428 that Aleppo pine populations in SE Spain had difficulties in recovering their functionality after  
429 the 2014 extreme drought event. Differences in vulnerability to embolism i.e. shape of  
430 vulnerability curves and derived P12, P50 and P88 parameters, did not explain the distinct  
431 mortality levels observed among populations. However, our findings show how these decline  
432 processes and mortality events correlated with the drought intensity at which trees were exposed.  
433 We also found that high levels of xylem embolism i.e. the PLC and negative HSM values  
434 observed in each population, were linked with higher tree mortality rates. In the populations  
435 located on the driest edge, where water scarcity conditions persisted, trees crossed hydraulic  
436 safety margins that promoted scarce growth and low carbohydrate content, and also favoured the  
437 impact of pest outbreaks. These partial contributions of different factors weakened the drought  
438 resistance of the different populations and, therefore, played an important role in triggering new  
439 mortality events and forest decline processes in following years.

440 ***Impact of drought on water potentials and hydraulic dysfunction.***

441 According to the SPEI values, the years herein considered correspond to a period of intense  
442 drought (González-Hidalgo et al. 2018). Consistently, the drought effect on Aleppo pine  
443 populations was more intense in those populations located on the driest edge of their distribution  
444 range (i.e. Orihuela, Albatera and San Juan) than those in the core of the species distribution  
445 range, such as Catí. The increase in the mean annual temperatures intensified the impact of water  
446 scarcity conditions. These persistent stressful conditions led water potential values to drastically  
447 drop up to -6 MPa at midday on days with high evaporative demand compared to normal records  
448 no lower than -3 MPa registered during normal precipitation regimes (Fotelli et al. 2019,  
449 Morcillo et al. 2019).

450 Thus vulnerability to xylem embolism (i.e. vulnerability curves and derived parameters), the PLC  
451 under drought conditions and the HSM have been previously reported as good predictors of plant  
452 mortality and forest decline (Adams et al. 2017, Benito-Garzón et al. 2018). However, our results  
453 do not show differences in vulnerability to xylem embolism among populations and it was not,  
454 therefore, related to the mortality levels observed in each population. Notwithstanding, the low  
455 water potentials measured at Orihuela meant that trees surpassed their hydraulic tolerance  
456 thresholds with high PLC values and showed narrow, or even negative, HSM values, which were  
457 associated with mortality. For this population, a critical PLC threshold of around 60% was  
458 established for determining mortality rates close to 100% in 2014. This PLC threshold is slightly  
459 higher than previous expectations for conifers, set at around 50% (Brodrribb and Cochard 2009),  
460 and comes closer to the more recent findings by Hammond et al. (2019) and Mantova et al.  
461 (2021), who reported lethal thresholds for conifers of ca. 80% and 70%, respectively. However,  
462 our results also revealed that not all mortality was attributable to PLC. After 2014, important  
463 mortality events continued in pine populations with lower PLC values, which highlights the role  
464 of other simultaneous factors in affecting survival and tree vigour. Our findings agree with recent  
465 studies about the limited capacity for predicting drought-induced tree mortality only by the  
466 hydraulics approach (Rowland et al. 2021, Venturas et al. 2021). In our study, drought legacy  
467 effects clearly impacted tree functioning in the years following 2014. These results agree with a

468 recent article (Mantova et al. 2022), which highlights that damage to key living tissues, e.g.  
469 meristems, determines the capacity to recover from drought.

470

#### 471 *Co-occurring processes linked with population weakening*

472 Consistently with the drought impact, both starch content and tree growth showed significant  
473 differences between populations after 2014, with lower values in those populations exposed to  
474 more stressful conditions. Previous studies point out that carbohydrate pools play an important  
475 role in plant vitality to resume growth and survival after drought (McDowell and Sevanto, 2010,  
476 Sevanto et al. 2014). Accordingly, the Albaterra and San Juan populations with the lowest starch  
477 levels showed less tree vigour. Diminished tree growth and leaf development have been  
478 commonly considered adaptative mechanisms to water shortage as they would help trees to  
479 improve the xylem's capacity to provide leaves with water (Maherali et al. 2002, Rosas et al.  
480 2013, Anderegg et al. 2019). However, growth reductions usually occur as a drought legacy effect  
481 (Gazol et al. 2018, Rodriguez-Vallejo et al. 2021) and can impair drought recovery in conifers  
482 (Brodribb et al., 2010), which may lead to tree mortality in forthcoming years (Camarero 2021).  
483 This increased mortality was observed especially for the San Juan population at the end of 2016.  
484 In fact San Juan and Albaterra had lower Ks values than for the other two populations. These lower  
485 Ks values may also be related to the persisting drought conditions beyond 2014, as reflected by  
486 the low water potential values like those found in the San Juan population late in the 2016-2017  
487 period, but also to the fact that still alive individuals were unable to resume xylem growth (Novak  
488 et al. 2016, Gričar et al. 2016). Moreover, these weaker conditions were exacerbated by biotic  
489 interactions, such as PPM and BB attacks, which promote leaf shedding, diminished  
490 photosynthetic capacity and, consequently, reduce carbohydrate production (Rodriguez-Vallejo  
491 et al. 2021). Although reductions in carbohydrate contents have not been directly associated with  
492 insect defoliations and damage (Castaño et al. 2020), they may promote alterations to phloem  
493 turgor, which would affect resin production and, therefore, trees' capacity to resist and survive  
494 pest infestations (Sevanto et al., 2014, Vázquez-González et al. 2020).

495

496 **Drought-induced forest decline: integrating tree responses into mortality**

497 We hypothesized that the most severely drought-affected populations would present higher PLC  
498 values, narrower HSMs and high mortality rates in the following years due to dieback processes  
499 and tree weakening. This hypothesis was partially supported by our results. According to the  
500 analysis of the integrated responses, there was a significant relation linking the 2014 mortality  
501 rates, drought stress levels in each population and the level of xylem embolism i.e. the PLC for  
502 those populations subjected to intenser drought conditions. In agreement with current postulates  
503 (Choat et al. 2018), the high xylem embolism values (i.e. PLC) and the negative HSM values in  
504 our study indicated that trees clearly suffered significant limitations to supply water to leaves,  
505 which consequently led to high mortality rates. In fact the mortality at Orihuela had almost  
506 affected the whole stand by the end of 2016 (*Figure S4 available as Supplementary Data at Tree*  
507 *Physiology Online*). The 2016 mortality values correlated with the tree vitality variables according  
508 to the RDA, such as low  $K_s$ , poor growth and leaf development, low starch content and a high  
509 degree of PPM infestation. These conditions were met specifically in the San Juan population  
510 during the post-drought period, where mortality had gone up to 80% by the end of 2016. These  
511 findings highlight the relevance of drought legacy effects on pine decline, but also the postdrought  
512 conditions in following years (Anderegg et al. 2015, DeSoto et al. 2020, Gazol et al. 2017). Apart  
513 from the important role played by xylem embolism in drought-induced tree mortality episodes,  
514 our results also underline the relevance of other factors, e.g. low carbohydrate and tree growth  
515 and biotic interactions, which affect tree vitality and promote forest decline, even in the  
516 populations that do not surpass the physiological thresholds, as in the San Juan population.  
517 Furthermore, the high temperatures recorded in 2014 and later may have favored more pest  
518 outbreaks, such as PPM, in the San Juan population, which weakened the individuals that  
519 remained alive (Vasconcellos & Duarte, 2018). Indeed the San Juan site also had the lowest starch  
520 contents, which would have significantly affected the capacity of these trees to resist such pest  
521 infestations (Castaño et al. 2020).

522 By way of conclusion, our research illustrates how severe drought events can promote a gradual  
523 loss of forest resilience in natural populations by affecting several key plant functional variables  
524 directly linked with trees' drought resistance, and, therefore, reduce both growth and survival  
525 capacity under drought conditions. Our results highlight the important role of the PLC and the  
526 HSM achieved by each population as predictors of tree survival under extreme drought  
527 conditions. However, our results also point out that a progressive decline process started after the  
528 2014 extreme drought, which led to subsequent pine tree death despite trees' lower water stress  
529 level, which is consistent with recent findings. This decline process has been related to low water  
530 transport capacity, poor plant growth, low carbohydrate content and marked pest impacts. All  
531 these factors should, therefore, be considered together when evaluating the impact of future  
532 droughts on these forests. Despite Aleppo pine being considered a well-adapted species to  
533 drought and other stressful conditions, our research results indicate that extreme drought events  
534 may negatively impact the resilience of this key species in the Mediterranean ecosystem in  
535 forthcoming years, especially under persistent dry conditions and in those populations closer to  
536 the climatic tolerance limit for this species.

537

538

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552

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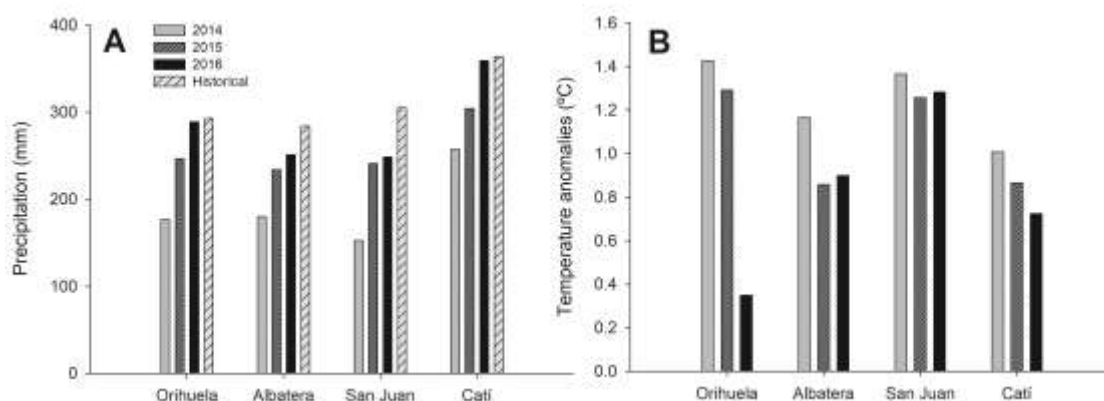
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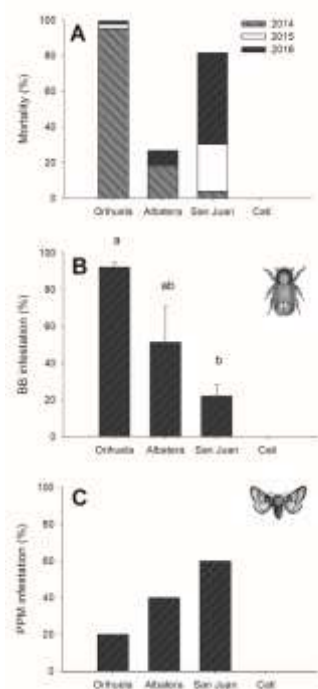
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799 **Figure legends**

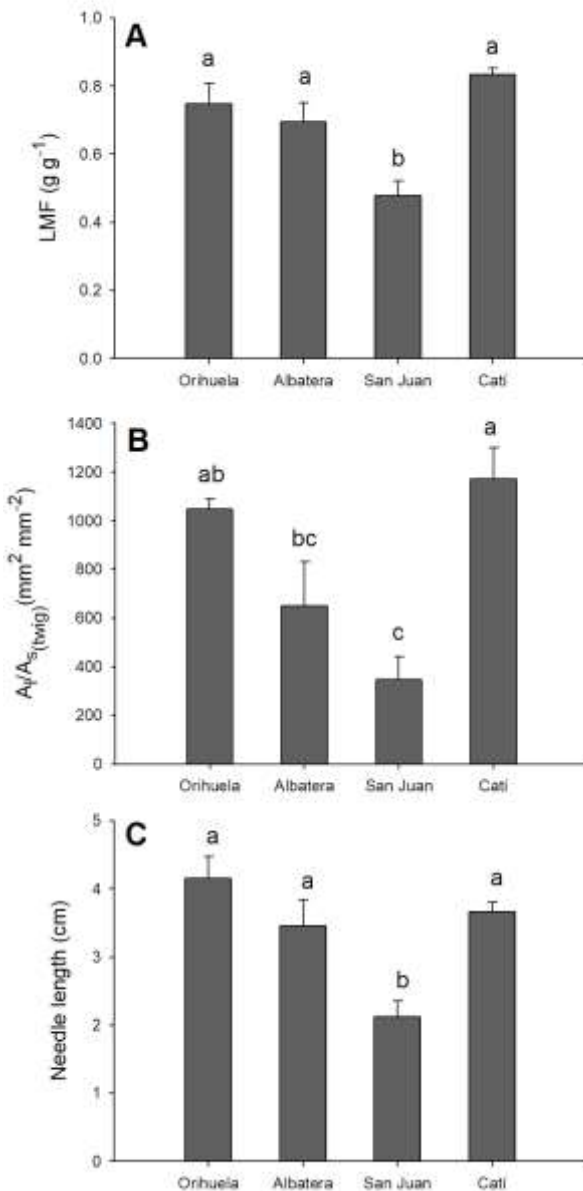
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 802 **Figure 1.** Annual precipitation in 2014 (light gray bars), 2015 (gray striped bars), 2016 (black  
 803 bars) and historical average precipitation from 1983 to 2012 (white striped bars) (A). Temperature  
 804 anomalies (difference between the annual average and the historical average) for each weather  
 805 station for 2014, 2015 and 2016 (B). Data were provided by the Spanish State Weather Agency  
 806 (AEMET) and the Valencian Institute of Agricultural Research (IVIA).  
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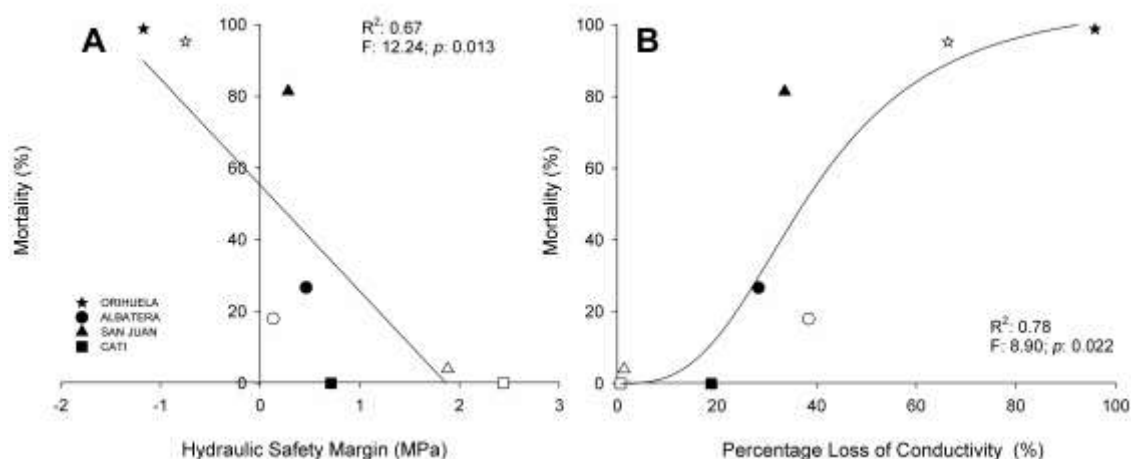
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 809 **Figure 2.** Percentage of pine mortality (A) recorded in 2014 (light gray striped areas), 2015 (white  
 810 areas) and 2016 (dark grey striped areas) in each population. The 2014 data were taken from  
 811 García de la Serrana et al. (2015). The percentage of dead pines affected by bark beetle attacks in  
 812 each population (B). Data correspond to dead pines at the end of 2016. Different letters mean  
 813 significant differences among populations according to the *post hoc* Tukey test at  $P < 0.05$ ;  $N=3$   
 814 plots in each population. The percentage of pine processionary moth (PPM) infestation in 2016  
 815 (C) according to the Regional Forestry Services survey in each population.



817

818 **Figure 3.** Leaf mass fraction, LMF (A), leaf to sapwood area ratio,  $A_l/A_s$  (B) and needle length  
 819 (C) in each pine population measured in November 2016. Different letters mean significant  
 820 differences among populations according to the *post hoc* Tukey test at  $P < 0.05$ ;  $N=7$ . Values are  
 821 the mean  $\pm$  SE (see Table S2, Suppl. Mat. for the complete statistics).

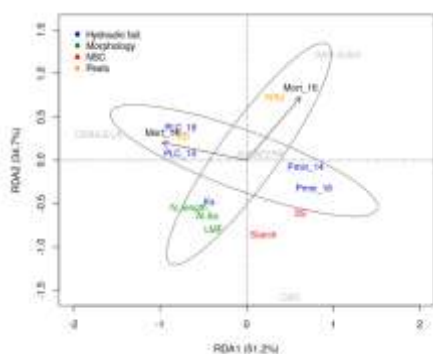
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824 **Figure 4.** Relation between mortality and the hydraulic safety margin (HSM) according to  $P_{50}$   
 825 (A) and the hydraulic dysfunction level (i.e. Percentage Loss of Conductivity, PLC) based on the  
 826 vulnerability curves for each population, fitted by an exponential relation (B). Mortality at all the  
 827 sites was recorded at the end of 2014 (from Garcia de la Serrana et al. 2015) and 2016. The  
 828 minimum water potentials to calculate the HSM and the PLC in 2014 were also taken from the  
 829 same manuscript. Values are represented by stars for Orihuela, dots for Albaterra, triangles for San  
 830 Juan and squares for Catí. Empty symbols correspond to the 2014 values and filled symbols to  
 831 2016. A shaded area highlights a high mortality threshold.

832



833

834 **Figure 5.** The redundancy analysis (RDA) of the 12 predictor variables: minimum water  
 835 potentials measured at midday ( $P_{min\_14}$  and  $P_{min\_16}$ ); hydraulic dysfunction level (i.e. PLC  
 836  $_{14}$  and PLC  $_{16}$ ); hydraulic conductivity ( $K_s$ ); needle length; leaf to sapwood area ratio ( $A_l/A_s$ );  
 837 leaf mass fraction (LMF); starch and soluble sugar contents (SS) and pest infestation (BB, bark  
 838 beetles; PPM, pine processionary moth) of the four Aleppo pine populations. RDA components  
 839 explained 86% variance (i.e. 51% and 35% for RDA1 and RDA2, respectively). Arrows denote  
 840 the principal components loadings for mortality.

841

842 **Table 1.** Minimum water potentials registered during the extreme drought in 2014 and during  
 843 the post-drought period (2015-17), and the main hydraulic parameters derived from the



844 vulnerability curves. P-values show the one-way ANOVA analysis results, for the population  
 845 effect on the variables  $P_{12}$ ,  $P_{50}$ ,  $P_{88}$ , and  $K_s$ . The significant differences between populations at  
 846 the  $P < 0.05$  level are highlighted in bold with different letters.

	$P_{\min\_2014}^*$	$P_{\min\_2016}$	$P_{12}$	$P_{50}$	$P_{88}$	$K_s (10^{-4})$
	-MPa	-MPa	-MPa	-MPa	-MPa	$\text{Kg} \cdot \text{m} \cdot \text{s}^{-1} \cdot \text{MPa}^{-1}$
Orihuela	-6.0±0.39 <sup>a</sup>	-6.4±0.39 <sup>a</sup>	4.5±0.14	-5.3±0.16	-6.0±0.19	3.7±0.71 <sup>ab</sup>
Albatera	-5.4±0.17 <sup>a</sup>	-5.1±0.32 <sup>b</sup>	-4.5±0.16	-5.6±0.18	-6.6±0.22	2.7±0.29 <sup>b</sup>
San Juan	-3.6±0.07 <sup>b</sup>	-5.2±0.16 <sup>b</sup>	-4.5±0.08	-5.5±0.08	-6.4±0.19	2.9±0.35 <sup>b</sup>
Catí	-3.2±0.13 <sup>b</sup>	-5.0±0.18 <sup>b</sup>	-4.7±0.11	-5.7±0.17	-6.7±0.23	4.9±0.53 <sup>a</sup>
$F_{\text{dof}}$	$F_{3,18}=54.1$	$F_{3,24}=5.76$	$F_{3,34}=0.47$	$F_{3,34}=1.42$	$F_{3,34}=1.95$	$F_{3,34}=3.56$
<b>P</b>	<b>&lt;0.001</b>	<b>0.004</b>	0.705	0.255	0.139	<b>0.024</b>

847 \* Data extracted from Garcia de la Serrana et al. 2015

848

849 **Table 2.** Average values of starch and soluble sugar (SS) contents ( $\text{mg} \cdot \text{g}^{-1}$ ) in July (2016) and  
 850 January (2017). Values are the average of seven samples per site ( $\pm$ SE). See Table S4 available  
 851 as *Supplementary Data at Tree Physiology Online* for the complete statistical analysis. The  
 852 significant differences between populations at  $P < 0.05$  level are highlighted with different letters.

		Orihuela	Albatera	San Juan	Catí
July 2016	Starch	0.84 ± 0.22 <sup>a</sup>	1.38 ± 0.19 <sup>a</sup>	0.63 ± 0.21 <sup>a</sup>	2.44 ± 0.34 <sup>b</sup>
	SS	1.63 ± 0.66	3.59 ± 1.06	2.86 ± 0.71	4.03 ± 1.02
January 2017	Starch	1.58 ± 0.23 <sup>ab</sup>	1.29 ± 0.08 <sup>bc</sup>	0.67 ± 0.11 <sup>c</sup>	2.12 ± 0.32 <sup>a</sup>
	SS	3.17 ± 0.87	1.86 ± 0.50	0.91 ± 0.51	3.45 ± 1.02

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854